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Original Article

Changes in $CD4^+CD25^+Foxp3^+$ Regulatory T Cells in Relation to Aging and Lung Tumor Incidence☆

Li-Jing Zhu [1], Pan-Fei Hou [2], Ling Wang [1]*, Guang-Bo Zhang [1], Yan Xie [1], Xu-Dong Pan [1], Ting-Ting Chang [1]

[1] *Clinical Immunology Laboratory, The First Affiliated Hospital of Soochow University, Jiangsu,* [2] *Rushan Hospital of Binzhou Medical University, Shandong, China*

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SUMMARY

Background: $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells mediate immunosuppression and play an important role in tumor immune evasion. Studies have demonstrated that this cell population represents an aging-related change. It is not clear whether this change leads to higher tumor incidence in the elderly. We investigated changes in $CD4^+CD25^+Foxp3^+$ Treg cells in relation to aging and tumor incidence.

Methods: We set up a Lewis lung cancer model with 26 C57BL/6 female mice. The animals were divided into six groups: young healthy, middle-aged healthy, elderly healthy, young tumor, middle-aged tumor and elderly tumor. We evaluated changes in $CD4^+CD25^+Foxp3^+$Treg cells in the spleen of all animals using a flow cytometry method. Levels of *Foxp3* m RNA in splenocytes were measured using a real-time RT-PCR method.

Results: The $CD4^+CD25^+Foxp3^+/CD4^+$ T cell ratio ($t = 2.23$, $p = 0.032$) and *Foxp3* mRNA levels ($t = 3.26$, $p = 0.0042$) were higher in the tumor groups than in the healthy groups. In the healthy groups, there was a significant increase in $CD4^+CD25^+Foxp3^+$ Treg cells on aging ($F = 47.70$, $p = 0.000$); elderly mice had a significantly greater population of $CD4^+CD25^+Foxp3^+$ Treg cells in spleen compared to the younger groups. The highest population was observed in the elderly tumor group. The same trend was evident for *Foxp3* mRNA ($F = 6.56$, $p = 0.0090$).

Conclusions: The results suggest a close relationship between changes in $CD4^+CD25^+Foxp3^+$ Treg cells and aging and lung tumor genesis and development.

1. Introduction

With aging, the incidence and prevalence of tumor increase[1–3], suggesting an intimate relationship between aging and tumors[4,5]. In other words, many mechanisms involved in the aging process share molecular mechanisms implicated in carcinogenesis[6]. The existence of relationships between aging and the risk of tumor development is not very well understood. From an immunology perspective, a tumor suppresses the immune response of the body and develops *in vivo* via many pathological processes, such as immunity escape, toleration and suppression[7]. Changes in the immunity system with aging further provide favorable conditions for tumor escape, so there is a higher incidence of tumors in the elderly. An understanding of the contribution of immunosenescence to tumor development and progression may help in designing better interventions for tumors in the elderly. Recent evidence demonstrated that regulatory T cell (Treg)-mediated immunosuppression is a crucial tumor immune evasion mechanism and contributes to the failure of tumor immunotherapy[8–11]. This regulatory capacities has been attributed to several different cell types, but the most characterized group is $CD4^+CD25^+Foxp3^+$ Treg cells, which increase with aging[12]. We presume that increases in $CD4^+CD25^+Foxp3^+$ Treg cells are responsible for the high tumor incidence in the elderly. Recently, lung tumor, as the highest rate of the malignant tumors[13], rises exponentially with aging[14]. In this study, we set up a Lewis lung cancer model in animals of different ages. We investigated changes in $CD4^+CD25^+Foxp3^+$ Treg cells in healthy and tumor model groups with aging and explored the relation between immunosenescence and lung tumors.

2. Materials and methods

2.1. Cell lines

Transplantable Lewis lung carcinoma (LLC) cells (Cell Bank, Shanghai Scientific Research Institute, Chinese Academy of Sciences)

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* Correspondence to: Dr Ling Wang, The First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China.
E-mail address: wangling40@126.com (L. Wang).

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were cultivated *in vitro* in DMEM culture medium with a high glucose content supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B in a 5% CO_2 atmosphere at 37 °C.

2.2. Animals and tumor model

C57BL/6 female mice ($n = 36$, Institute of Laboratory Animals, Shanghai SLAK, Certificate of Quality No. 2007000504834) were divided into six groups: 4-week healthy (young healthy, YH), 6-month healthy (middle-aged healthy, MH), 20-month healthy (elderly healthy, EH), 4-week tumor (young tumor, YT), 6-month tumor (middle-aged tumor, MT) and 20-month tumor (elderly tumor, ET), with six mice in each group. Mice were maintained under specific pathogen-free conditions and provided with food and water *ad libitum*. All animals were housed for 1 week and maintained under standard conditions prior to experimentation. All animal procedures were performed in accordance with the rules of the local Ethics Committee and the guidelines established by the Animal Care Committee, which approved the study (No. 2091116). All tumor groups were injected subcutaneously with 0.1 mL of LLC cells (2×10^6 cells) in the left forelimb. The healthy groups were injected with the same amount of phosphate-buffered saline (PBS) in the same site. After implantation, tumor growth dynamics was monitored every 2 days. The tumor long diameter (L) and short diameter (W) were used to calculate tumor volume according to the Steel formula: $V = LW^2/2$. Tumor growth curves were plotted for all experimental groups.

2.3. Flow cytometry

The mice were sacrificed 20 days after the injection treatments. The spleen was harvested and a splenocyte suspension was prepared. Lymphocytes were isolated from this suspension by density centrifugation over a Ficoll-Hypaque gradient (Lymphoprep, Tian Jin Hao Yang Biological Manufacture, Tianjin, China). An aliquot of 100 μL (1×10^6 cells) was removed for dyeing, and 1 mL of RNAiso Plus was added to the remainder for preservation at −80 °C for measurement of *Foxp3* mRNA.

A Mouse Regulatory T cell staining kit (ebioscience, San Diego, USA) was used for staining according to the manufacturer's directions. In brief, 100 μL of prepared cells (1×10^6) was added to individual tubes and surface molecules were stained using a standard procedure, with a fluorescein isothiocyanate-CD4 antibody and an allophycocyanin-conjugated CD25 antibody. After washing with flow cytometry staining buffer, cells were treated with fixation/permeabilization working solution and washed once with 2 mL of permeabilization buffer. After centrifugation, the supernatant was decanted and cells were then stained with a phycoerythrin-conjugated Foxp3 antibody. Finally, cells were washed with 2 mL of permeabilization buffer, resuspended in flow cytometry staining buffer, and analyzed by flow cytometry (Beckman Coulter, Inc. California, USA).

2.4. Real-time RT-PCR

Spleen cells were isolated and preserved at −80 °C. DNA-free RNA templates were prepared using an RNA isolation kit (Shinegene Bio-Technologies, Shanghai, China). RNA concentrations and quality were assessed using spectrophotometry at wavelengths of 260 and 280 nm. RNA was reverse-transcribed using a reverse transcription kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. Real-time PCR assays were carried out using a SYBR® Premix Ex Taq™ kit (Takara Biotechnology). The 20-μL reaction system contained SYBR Premix Ex Taq (10 μL), ROX Reference Dye II (0.4 μL), Foxp3 primers (5′-CAG CTG CCT ACA GTG CCC CTA G-3′ and 5′-CAT TTG CCA GCA GTG GGT AG-3′, 0.4 μL each) cDNA (2 μL) and dH_2O (6.8 μL). PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Inc. California, USA). The conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. HPRT (5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ and 5′-GAT TCA ACT TGC TCT CAT CTT AGG C-3′) was used as a housekeeping gene for normalization of gene transcript levels.

2.5. Statistical analysis

Values are presented as mean ± standard deviation (SD). Statistical analysis was performed using SAS version 8.0 software. Student's t test was used to examine the significance of differences between two groups. One-way analysis of variance (ANOVA), followed by Dunnet's test and a q test, was used to compare means across multiple groups. A p value of < 0.05 was deemed to be statistically significant.

3. Results

3.1. Establishment of the tumor model

We applied a classic approach to establish a lung tumor model. After injection of LLC cells, tumors were palpable on the 10th day and could be seen by the naked eye on the 16th day. Tumor growth curves are shown in Fig. 1. By contrast, the healthy groups showed no changes. All the mice were killed 20 days after implantation (Fig. 2) and splenocytes were isolated and used in experiments.

3.2. Changes in $CD4^+CD25^+Foxp3^+$ Treg cells in spleen

The presence of $CD4^+CD25^+Foxp3^+$ Treg cells was evaluated in spleen for the six groups. As shown in Fig. 3 and Table 1, the ratio

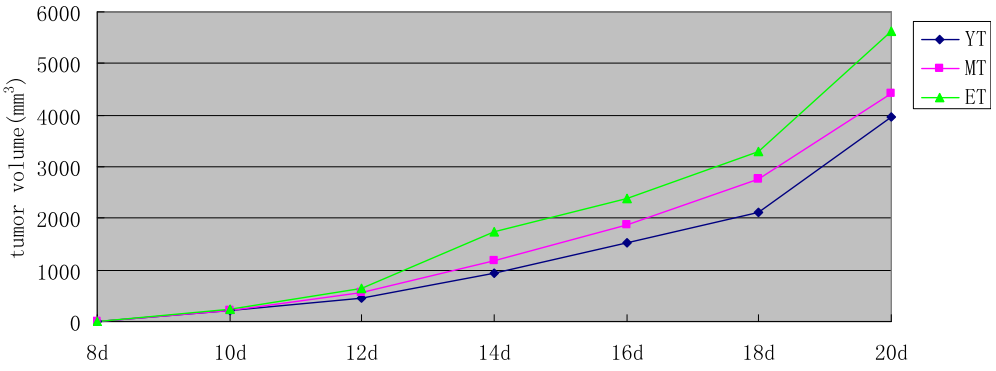

Fig. 1. Tumor growth curves for the experimental groups.

Fig. 2. A 4-week-old mouse sacrificed 20 days after injection. Tumor tissue in the left forelimb is clearly evident.

of $CD4^+CD25^+Foxp3^+$ to $CD4^+$ T cells was higher in the tumor groups than in the healthy groups ($t = 2.23$, $p = 0.032$). The ratio showed significant differences among the healthy groups ($F = 47.70$, $p = 0.000$). The MH group had a larger population of $CD4^+CD25^+Foxp3^+$ Treg cells in spleen compared to the YH group ($F = 53.35$, $p = 0.0019$) and there was a significant difference between the EH and MH groups ($F = 22.02$, $p = 0.0082$). The highest ratio was observed for the ET group.

Table 1
Comparison of the $CD4^+CD25^+Foxp3^+/CD4^+$ cell ratio in spleen for the six groups.

Group	Healthy	Tumor
Young (4 w)	1.56 ± 0.43	2.13 ± 0.33
Middle-aged (6 m)	2.60 ± 0.30*	3.25 ± 0.42
Elderly (20 m)	3.71 ± 0.40**	5.01 ± 0.24
F values	47.70	108.29
P values	<0.01	<0.01

* $p = 0.0019$ compared with the young healthy, ** $p = 0.0082$ compared with the middle-aged healthy group.

3.3. *Foxp3* mRNA expression levels in spleen

We investigated the levels of *Foxp3* mRNA for the six groups. *Foxp3* mRNA amplification was successfully performed by RT-PCR (Fig. 4). Our results revealed differences among the six groups (Fig. 5). *Foxp3* mRNA levels were higher in the tumor compared to the healthy groups ($t = 3.26$, $p = 0.0042$). *Foxp3* mRNA levels in spleen also increased with aging ($F = 6.56$, $p = 0.0090$); among the healthy groups, levels were significantly lower in the YH group compared to the MH and EH groups. In agreement with its immune function, changes in *Foxp3* expression were consistent with variations in $CD4^+CD25^+Foxp3^+$ Treg cells. Levels of *Foxp3* mRNA were affected by both age and tumor incidence, and the highest levels were found in the elderly tumor group.

4. Discussion

The decrease in activation of cell-mediated immune responses with aging may be related to the age-associated increase in tumor incidence[15–17]. Besides CD28 and CD95[18], Treg cells play a crucial role. $CD4^+CD25^+Foxp3^+$ Treg cells show high expression of cell-surface molecules, including CD25, cytotoxic T-lymphocyte-associated protein-4 (CTLA4) and glucocorticoid-induced tumor necrosis factor receptor (GITR), and are characterized by expression

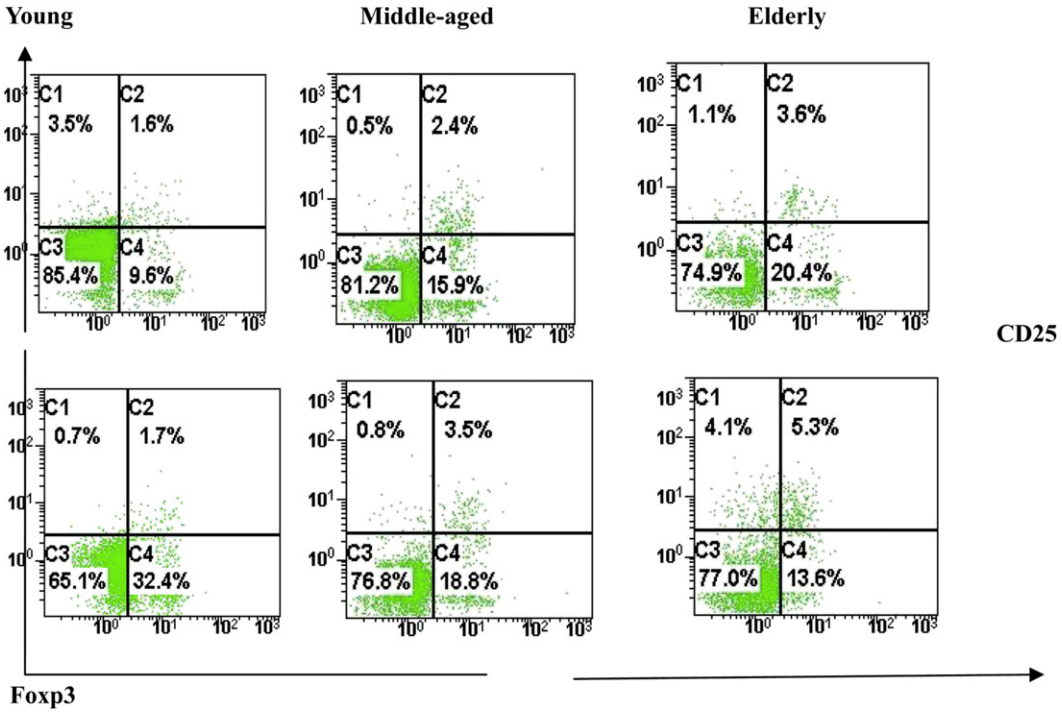

Fig. 3. Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ Treg cells for the six groups. Representative FACS analyses of $CD4^+$-cell-gated spleen preparations from the six groups are presented. The top row represents the healthy group, and the bottom row the tumor group.

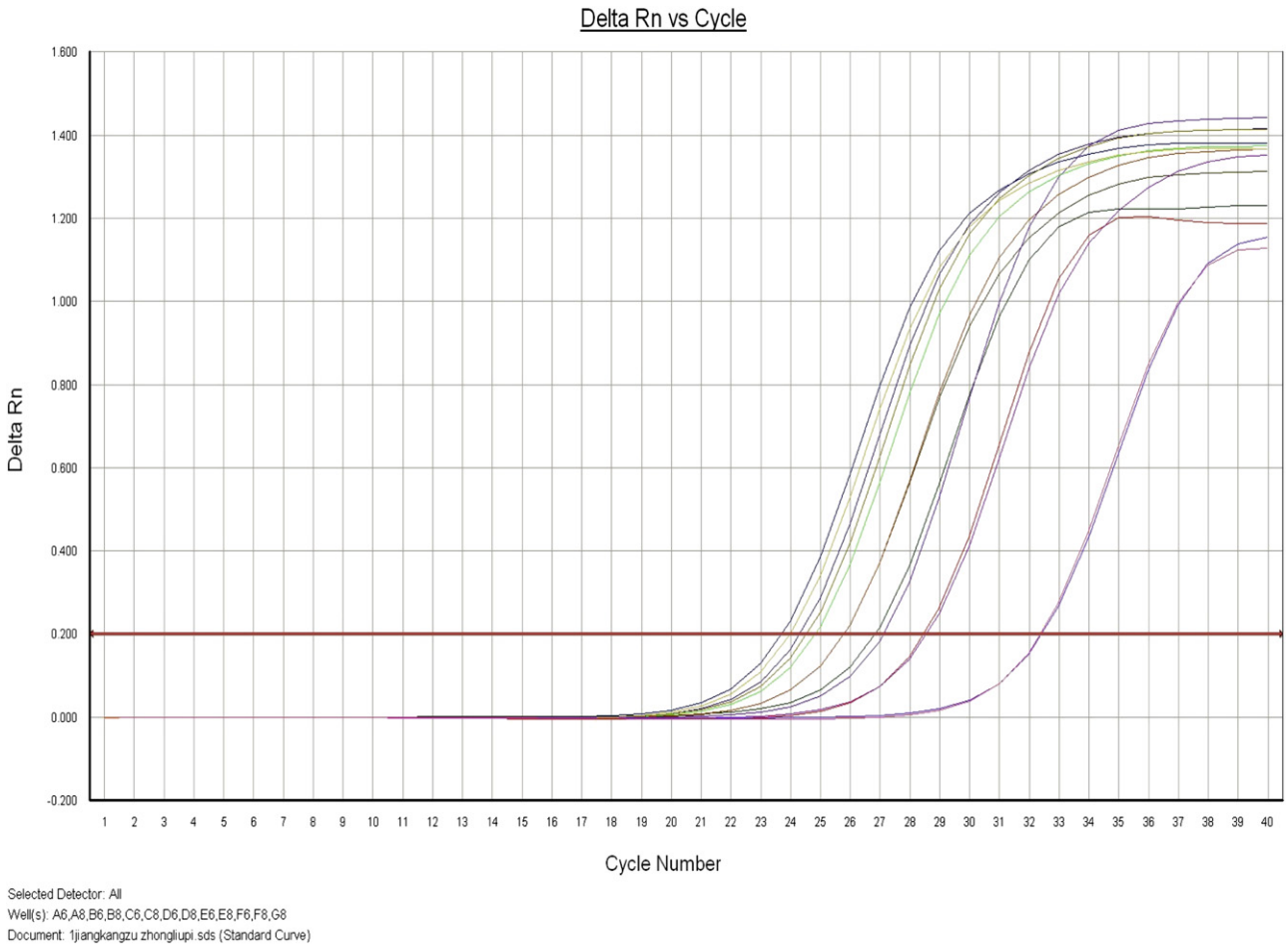

Fig. 4. Gene amplification for *Foxp3* was carried out successfully by RT-PCR.

of a master regulator gene, forkhead transcription factor 3 (Foxp3)[19,20]. The constitutive expression of these factors is involved in immunosuppression via several molecular mechanisms. CTLA4 interacts with CD80 and CD86 co-stimulatory molecules expressed by antigen-presenting cells (APCs) and transduces a suppressive signal[21,22]. Alternatively, it can directly mediate suppression by triggering induction of the enzyme indoleamine 2,3-dioxygenase in dendritic cells (DCs), which catalyzes the conversion of tryptophan to kynurenine and other metabolites and has potent immunosuppressive effects on DCs. GITR is also present on the surface of $CD4^+CD25^+Foxp3^+$ Treg cells and its ligand is expressed on APCs in mice and on plasmacytoid DCs in human; much evidence indicates that it plays a role in suppressing effector T cells[23,24]. The

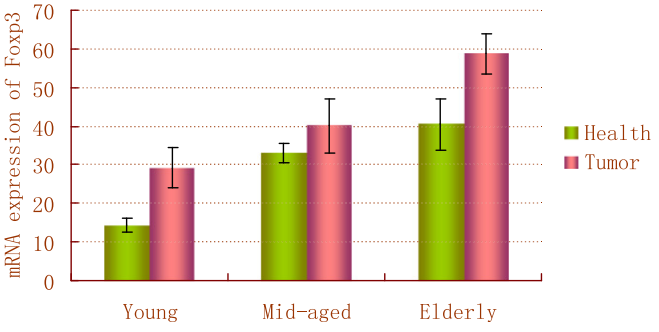

Fig. 5. *Foxp3* mRNA expression in spleen from the six groups. Data are mean ± SD values after normalization to *HPRT* levels.

transcription factor Foxp3 is a key player in conferring a suppressive function to $CD4^+$ T cells[25,26]. Retroviral transduction of Foxp3 upregulates Treg-associated molecules, including CD25, CTLA4 and GITR, and suppresses gene transcription for cytokines such as IL-10 and TGF-β[20]. Besides mechanisms that depend on cell—cell contact, $CD4^+CD25^+Foxp3^+$ Treg cells are involved in the maintenance of downregulation of various immune responses by these cytokines in an indirect manner[27].

In the present study, we set up a Lewis lung tumor model in mice of different ages and analyzed changes in the number of $CD4^+CD25^+Foxp3^+$ Treg cells and in *Foxp3* mRNA levels in splenocytes. Our results revealed significant increases in both parameters for tumor groups compared with the corresponding healthy groups, which is consistent with previous studies[28,29]. In the tumor groups, as a result of immunosuppression, the increase in $CD4^+CD25^+Foxp3^+$ Treg cells further supported tumor immune evasion, whereas other studies demonstrated no correlation between a higher number of $CD4^+CD25^+Foxp3^+$ Treg cells and suppression activity[30,31]. It is not known precisely why $CD4^+CD25^+Foxp3^+$ Treg cells are more numerous in mice with tumors compared to their healthy counterparts. Research indicates that natural $CD4^+CD25^-$ T cells are converted to Treg cells by IL-10 and TGF-β, which are tumor-derived soluble factors, under tumor conditions[32,33].

Results for the healthy groups revealed that the number of the $CD4^+CD25^+Foxp3^+$ Treg cells and *Foxp3* mRNA levels increased with aging. Despite a wealth of studies, there are no obvious explanations of why the $CD4^+CD25^+Foxp3^+$ Treg cell population

increases with aging in healthy individuals and where it comes from. Levels of both IL-10[34] and TGF-β[35] increase with aging, and $CD4^+CD25^+Foxp3^+$ Treg cells in elderly mice might be generated from $CD4^+CD25^-Foxp3^-$ T cells, induced by changes in TGF-β and IL-10 in a tumor environment. Another important explanation is the existence of two subsets of $CD4^+CD25^+Foxp3^+$ Treg cells with very distinct fates, as confirmed by Fisson et al[36]. One subset exhibits a rapid proliferation rate characterized by a $CD44^{high}$ phenotype, whereas the other subset does not divide but is long-lived, and is characterized by a $CD44^{int}$ phenotype. These two subsets might be relevant to the increase in number, but the mechanism has not been completely clarified.

Our experimental results reveal a close relationship between changes in $CD4^+CD25^+Foxp3^+$ Treg cells and aging and lung tumor genesis and development. $CD4^+CD25^+Foxp3^+$ Treg cells play an important role in inhibiting immune responses, and increases in this cell population with aging reduce the activity of the immune system. Thus, the increase in $CD4^+CD25^+Foxp3^+$ Treg cells with aging may be a major factor in tumor incidence and the damage caused by this increase may translate into a susceptibility to tumorigenesis in the elderly. Evaluation of changes in $CD4^+CD25^+Foxp3^+$ Treg cells with aging to establish a normal range for these cells at different ages could help in providing a threshold target for immune crises in the elderly and in predicting tumor occurrence. Furthermore, if questions related to the mechanism involved, such as immunological tolerance and accumulation, can be answered, it should be possible to take effective measures to interfere with this mechanism, which would open new prospects for Treg-targeted immunomodulatory therapy.

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